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Human topoisomerase IIα (*hs*topo IIα) is an essential enzyme that is the target of a number of anticancer drugs in clinical use. Clinically, resistance to anticancer drugs develops through various mechanisms, one of which can be found in a class of atypical multidrug resistant mutants (at-MDR) of *hs*topo IIα. Our goal is to clarify the biochemical basis of at-MDR among *hs*topo IIα mutants to shed more light on the topo II enzymatic mechanism. Additionally, we have identified sites of covalent thiol modification of *hs*topo IIα by anticancer drugs and chemopreventive agents. To this end, we developed a cysteine footprinting technique using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to assess the differential reactivities of the cysteine residues of *hs*topo IIα and compared their relative solvent accessibilities to a solvent contact surface (SCS) analysis of a structural model of *hs*topo IIα. Experiments are in progress to evaluate the effects of DNA, nucleotide cofactors, and at-MDR mutants on the relative thiol reactivities of *hs*topo IIα. Additionally, using LC-ESI-MS, we have located sites of covalent cysteine modification of *hs*topo IIα by both anticancer drugs and chemopreventive agents, resulting in the first direct evidence of this novel poisoning mechanism by thiol alkylation of *hs*topo IIα.

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## Introduction

We are interested in the mechanism of *hs*topoIIα because it is the target of several drugs currently being used in cancer therapy such as etoposide and doxorubicin (Harris and Hochhauser 1992; Isaacs, Davies et al. 1995; Burden and Osheroff 1998). A number of these drugs work by trapping a DNA cleavage intermediate in which topo II is covalently attached to DNA. As a result, these drugs render the enzyme nucleolytic, inducing cytotoxicity. However, cancer cells become resistant over time (Harris and Hochhauser 1992). A unique class of at-MDR mutants has been discovered, some of which are characterized by a single amino acid change in topo II. These amino acid substitutions have been mapped to the Gyr B' and Gyr A' domains (Mao, Yu et al. 1999; Wang, Mao et al. 2001) While some of these mutants have undergone initial characterization, very few biochemical experiments have been carried out on at-MDR mutants of *hs*topo IIα. We will attempt to further clarify the biochemical basis of at-MDR among *hs*topo IIα mutants to shed more light on the topo II enzymatic mechanism. To accomplish this goal, we have employed protein footprinting at cysteine residues using an alkylation/endoproteinase procedure coupled with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Structural changes of *hs*topo IIα will likely lead to an alteration in the solvent-accessibility of the protein surface as domains open and close, resulting in varied sensitivity of amino acid residues towards footprinting reagents. This will allow us to probe conformational changes of wild-type and at-MDR mutants of *hs*topo IIα in the presence and absence of anticancer drugs, DNA, and cofactors in order to further understand the *hs*topo IIα mechanism and the biochemical basis of resistance among at-MDR mutants.

Using LC-ESI-MS, we will also attempt to further demonstrate how newly identified topo II poisons and chemopreventive agents act to target the enzyme. Studies have shown that topo II can be poisoned by various mechanisms. (Liu, Rowe et al. 1983; Zechiedrich, Christiansen et al. 1989; Frydman, Marton et al. 1997; Kwok and Hurley 1998). A mechanism for topo II poisoning was recently discovered in which thiol alkylation of topo II stimulates topo II-dependent DNA cleavage (Frydman, Marton et al. 1997; Wang, Mao et al. 2001). A cysteineless mutant yeast topo II was found to be completely resistant to thiol-alkylating drugs, which implicates cysteine modification in the mediation of topo II-directed DNA cleavage (Wang, Mao et al. 2001). We have attempted to map the specific cysteine residue(s) involved in this mechanism by using an endoproteinase/mass spectrometry footprinting approach.

## Body

*Protein Conformational Changes*

To address the questions at hand, we have made progress towards accomplishing our research goals during the past year. Previously, recombinant *hs*topo IIα was created by fusing an HMK site and $H_6$ tag to the N-terminus of *hs*topo IIα. HMK-$H_6$-*hs*topo IIα was purified by affinity and ion exchange chromatography; the purified protein displayed activity comparable to the wild type protein. The aforementioned construct was generated for the footprinting procedure initially developed during the previous year, in

which HMK-$H_6$-*hs*topo IIα was treated with NTCB and subjected to alkaline denaturing conditions resulting in a ladder of peptide fragments which were radiolabeled, separated by SDS-PAGE and visualized by phosphorimaging. However, due to the difficulty encountered with the NTCB cysteine footprinting procedure, attempts were made to find an alternative footprinting procedure that could answer the same questions we began with. However, the HMK-$H_6$-*hs*topo IIα construct was retained for use in the current development of an alternative cysteine footprinting approach.

To this end, an endoproteinase footprinting procedure utilizing mass spectrometry was developed. Initial experiments were targeted at successfully identifying cysteine-containing peptides from a tryptic digest mixture by comparing spectra of reduced samples to those alkylated with iodoacetamide (IAM). For identification of alkylated cysteine-containing peptides, HMK-$H_6$-*hs*topo IIα was treated with IAM and denatured. Upon removal of excess denaturant and IAM, the sample was completely digested with trypsin and subjected to LC-ESI-MS. This sample was then compared to that of a tryptic digest of HMK-$H_6$-*hs*topo IIα which was not alkylated with IAM. The results from these experiments resulted in the successful identification of cysteine-containing peptide fragments covalently modified by IAM (Table 1).

| **Predicted Tryptic Peptide Fragment (sequence number range)** | **$MH_x^{x+}$ Calc. for Iodoacetamide Alkylation** | **$MH_x^{x+}$ Exp. for Iodoacetamide Alkylation** |
|---|---|---|
| | *m/z* | *m/z* |
| MSCIR (102-106) $^{x=2}$ | 333.6531 | 333.6749 |
| LCNIFSTK (169-176) $^{x=2}$ | 491.7513 | 491.7631 |
| AGEMELKPFNGEDYTCITFQPDLSK (201-225) $^{x=4}$ | 723.3318 | 723.3589 |
| WEVCLTMSEK (297-306) $^{x=2}$ | 641.7903 | 641.8027 |
| SFGSTCQLSEK (387-397) $^{x=2}$ | 622.2812 | 622.2937 |
| AAIGCGIVESILNWVK (401-416) $^{x=2}$ | 865.4653 | 865.5059 |
| CSAVK (427-431) $^{x=2}$ | 282.6405 | N.F. |
| NSTECTLILTEGDSAK (451-466) $^{x=2}$ | 520.2721 | 580.2914 |
| VLFTCFK (729-735) $^{x=2}$ | 457.7402 | 457.7411 |
| VEPEWYIPIIPMVLINGAEGIGTGWSCK (836-863) $^{x=3}$ | 1043.8596 | 1043.8671 |
| LQTSLTCNSMVLFDHVGCLK (991-1010) $^{x=3}$ | 775.0414 | 775.0721 |
| DELCR (1142-1146) $^{x=2}$ | 346.6516 | N.F. |

**Table 1**: Predicted and experimental mass values for the 13 cysteine-containing tryptic peptides. X+ represents the charge state of the major ionic species observed. m/z = mass to charge ratio, N.F. = not found

Once it was established that we could identify all of the tryptic peptides containing cysteine residues, we began assessing the differential reactivities of the cysteines of HMK-$H_6$-*hs*topo IIα. However, quantification of the alkylated cysteine-containing peptides is necessary for the validity of our approach. Thus, we developed a quantitiative mass spectrometric footprinting procedure utilizing both IAM as well as

deuterated iodoacetamide (D-IAM). Equivolume mixtures containing varying molar proportions of D-IAM and IAM modified peptides were mixed and analyzed by LC-ESI-MS. The quantitative nature of the procedure was verified by the linear relationship obtained from a plot of the relative peptide abundance (the percentage of intensity of the D-IAM modified peak divided by the sum of intensities of both D-IAM and IAM modified peaks = $P_{D\text{-}IAM} / (P_{D\text{-}IAM} + P_{IAM})$) versus its relative concentration ratio. Once the quantitative nature of the procedure was confirmed, D-IAM and IAM were used to evaluate the relative thiol reactivities of HMK-$H_6$-*hs*topo IIα. To this end, the enzyme was initially treated with various concentrations of D-IAM and was subsequently denatured and allowed to react with excess IAM. The protein was then dialyzed and digested with trypsin to create peptide fragments that were subjected to LC-ESI-MS. Because the protein was first treated with D-IAM before being denatured, a larger extent of D-IAM modification indicates elevated thiol reactivity, which likely correlates with increased solvent accessibility. For example, if a particular residue is located on the exterior of *hs*topo IIα resulting in high solvent accessibility, it is likely to react to a larger extent with the first reagent, D-IAM. More buried thiols may be less solvent accessible, resulting in less reaction with D-IAM, but increased reaction with IAM because this reagent is present after the protein has been denatured. Based on the relative ratios of cysteine-containing peptide modification with D-IAM versus IAM, 11 cysteines were divided into three groups according to their order of reactivity: Cys104 > Cys733, Cys170, Cys392, Cys300, Cys455, Cys405, Cys216 > Cys997-1008, Cys862 (Figure 1).

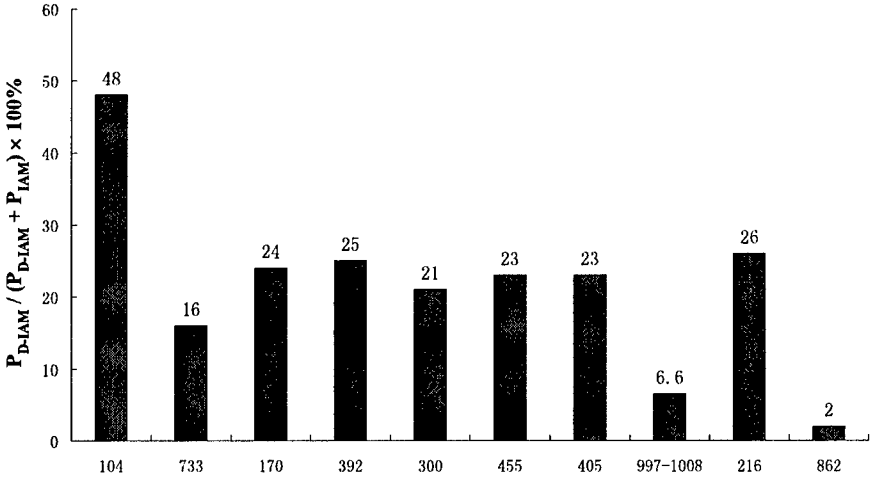


**Figure 1:** Percentages of D-IAM modification for different cysteines at 0.2 mM D-IAM

To supplement and substantiate the biochemical data described thus far, an *hs*topo IIα structural model was constructed using *S. cerevisiae* topoisomerase II as a template. (Figure 2). Solvent contact surface analysis of the *hs*topo IIα homology model revealed four cysteines, Cys1008, Cys104, Cys216, and Cys405, as being the most solvent-accessible. These data agreed with that from the footprinting experiments, with the exception of Cys1008, which was shown to be one of the least reactive residues by our experimental data. One possible reason for this disagreement is that a sequence of 63

residues near Cys1008 was not modeled due to lack of *S. cerevisiae* template structure in this region. It is possible that the missing 63 amino acids actually shielded Cys1008 from the solvent and thus made it less accessible. However, the *hs*topo IIα homology model appears to be a useful tool for visualizing the position of each thiol relative to the global structure of *hs*topo IIα. The model should prove to be a valuable visual aid and complement to L-ESI-MS footprinting. Experiments are in progress to probe the conformation of *hs*topo IIα and at-MDR mutants in the presence of DNA, ATP, and other cofactors, thus offering further insight into its structural dynamics.

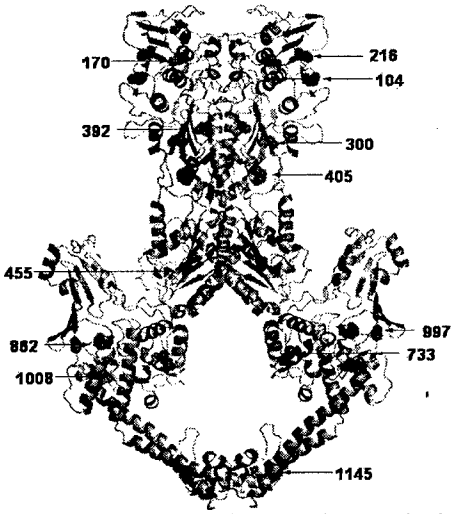


**Figure 2:** Model of Human topoisomerase IIα, with 12 cysteines emphasized in red. Cys427 was not modeled because corresponding structure was not present in the *S. cerevisiae* template.

*Cysteine Binding sites*

Using mass spectrometry, we have also identified sites of thiol alkylation of *hs*topo IIα by both anticancer drugs such as menadione and chemopreventive compounds such as diallyl trisulfide (DAT), which has been shown to induce topoisomerase II-mediated DNA cleavage. In this procedure, we directly observed thiol modification by identifying the appearance of new tryptic peptide fragments in alkylated samples. In this procedure,

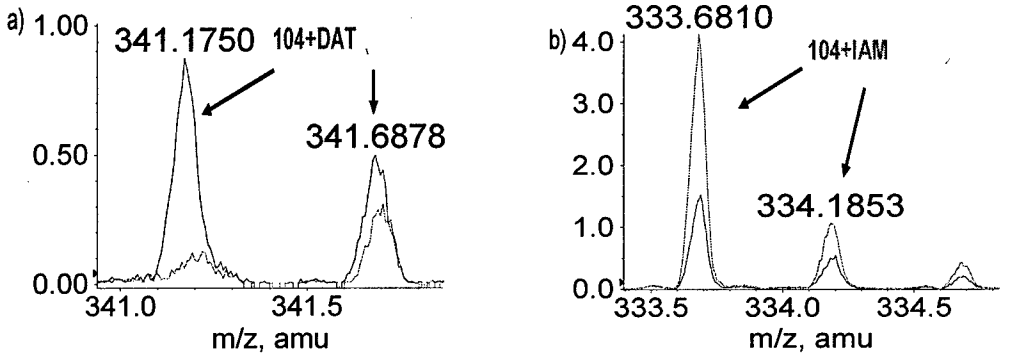


**Figure 3:** Mass Spectra demonstrating the modification of Cys104 by DAT a) Direct evidence: appearance of a novel peak occurs in the DAT-treated sample (blue, upper spectrum), but is not present in the untreated sample (red, lower spectrum). b) Indirect evidence: the cysteine-containing peptide MSCIR pretreated with DAT (blue, lower spectrum) was not modified with IAM to the same extent as the sample not treated with DAT (red, upper spectrum) because it had previously reacted with DAT, preventing reaction with IAM. Arrows indicate different isotopes of the peptide, which are in the 2+ charged state.

HMK-$H_6$-*hs*topo IIα was incubated in the presence or absence of DNA and anticancer drugs or chemopreventive compounds. The reaction was quenched and HMK-$H_6$-*hs*topo IIα was treated with excess IAM followed by trypsin digestion and LC-ESI-MS. In this procedure, it is feasible to discover sites of covalent modification by DAT, for example, by identifying fragments in DAT-treated samples that are not present in untreated samples. If the unique fragment corresponds to a molecular weight increase consistent with a reaction between DAT and a cysteine residue, then the site of modification can be identified. Moreover, the IAM-modified peptide will decrease in intensity or disappear altogether for samples that were pretreated with DAT, because their reaction with DAT precludes their reaction with IAM. Our preliminary data provides direct evidence that the chemopreventive compound DAT is reacting with thiol groups on HMK-$H_6$-*hs*topo IIα. For example, as seen in Figure 3a, treatment with DAT results in the appearance of a new fragment consistent with a reaction between DAT and Cys104 contained in the peptide fragment MSCIR. Additionally, in the sample pretreated with DAT, the amount of IAM-modified peptide decreased (Figure 3b) because Cys104 had reacted with DAT, the first reagent it encountered, resulting in less reaction with the second reagent added, IAM, and hence a diminished signal for the IAM-modified peptide fragment. While data from LC-ESI-MS suggests that DAT modifies Cys104 to the greatest extent, additional thiols are modified as well. Thus, experiments are in progress to help determine the cysteine residues that are critical for this novel poisoning mechanism of *hs*topo IIα; site directed mutagenesis coupled with *in vitro* DNA cleavage assays will help to answer these remaining questions.

During the previous year, we putatively identified menadione as having reacted with Cys427 by matrix-assisted laser desorption ionization (MALDI) MS. Preliminary results from LC-ESI-MS suggest that menadione reacts with additional thiol residues, albeit through indirect evidence. The indirect evidence is similar to that mentioned previously in which samples first treated with menadione followed by treatment with IAM exhibited diminished signals for cysteine-containing peptides modified with IAM, suggesting prior reaction with menadione precluding reaction with IAM. Additionally, chemopreventive compounds such as that found broccoli, benzyl isothiocyanate (BITC), have been tested, and indirect evidence also suggests reaction with thiol residues. Experiments are in progress to test a range of potential topoisomerase II poisons that act to inhibit the enzyme by thiol alkylation.

## Key Research Accomplishments

- Successful development and implementation of an alternative footprinting technique using LC-ESI-MS
- Reproducible identification of tryptic peptides containing cysteine residues
- Establishment of the relative thiol reactivity of *hs*topo IIα in native conditions
- Development of structural model of *hs*topo IIα based on homology to the *S. cerevisiae* topoisomersase II structure
- Identification of thiol modification sites by anticancer drugs and chemopreventive compounds
- Putative verification of a new means by which thiol alkylators poison *hs*topo IIα

## Conclusions

Human topoisomerase IIα is an essential enzyme that is the target of a number of anticancer drugs in clinical use, making the understanding of its catalytic mechanism very important. Thus, we are examining the mechanism of *hs*topo IIα using mass spectrometric cysteine footprinting in a procedure utilizing tryptic digestion coupled with LC-ESI-MS. Using LC-ESI-MS cysteine footprinting, we have assessed the differential thiol reactivities of the cysteine residues of *hs*topo IIα. To complement the thiol reactivity results obtained by LC-ESI-MS, we have compared our data to a solvent contact surface analysis of an *hs*topo IIα structural model based on homology with its yeast counterpart. Moreover, we have found direct evidence of covalent thiol modification of *hs*topo IIα using LC-ESI-MS. These results exhibit the first direct evidence that thiol alkylation is indeed a novel mechanism of *hs*topo IIα poisoning. In the future, we will further clarify the biochemical basis of resistance among at-MDR mutants and we expect to complement our drug binding data with site-directed mutagenesis to pinpoint those cysteines most important in the thiol alkylation poisoning mechanism of *hs*topo IIα.

## References


Burden, D. A. and N. Osheroff (1998). "Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme." Biochim Biophys Acta **1400**(1-3): 139-54.

Frydman, B., L. J. Marton, et al. (1997). "Induction of DNA topoisomerase II-mediated DNA cleavage by beta-lapachone and related naphthoquinones." Cancer Res **57**(4): 620-7.

Isaacs, R. J., S. L. Davies, et al. (1995). "Topoisomerases II alpha and beta as therapy targets in breast cancer." Anticancer Drugs **6**(2): 195-211.

Harris, A. L. and D. Hochhauser (1992). "Mechanisms of multidrug resistance in cancer treatment." Acta Oncol **31**(2): 205-13.

Kennelly, P. J. and E. G. Krebs (1991). "Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases." J Biol Chem **266**(24): 15555-8.

Kwok, Y. and L. H. Hurley (1998). "Topoisomerase II site-directed alkylation of DNA by psorospermin and its effect on topoisomerase II-mediated DNA cleavage." J Biol Chem **273**(49): 33020-6

Liu, L. F., T. C. Rowe, et al. (1983). "Cleavage of DNA by mammalian DNA topoisomerase." The Journal of Biological Chemistry **258**: 15365-15370.

Mao, Y., C. Yu, et al. (1999). "Mutations of human topoisomerase II alpha affecting multidrug resistance and sensitivity." Biochemistry **38**(33): 10793-800.

Wang, H., Y. Mao, et al. (2001). "Stimulation of topoisomerase II-mediated DNA damage via a mechanism involving protein thiolation." Biochemistry **40**(11): 3316-23.

Zechiedrich, E. L., K. Christiansen, et al. (1989). "Double-stranded DNA cleavage/religation reaction of eukaryotic topoisomerase II: evidence for a nicked DNA intermediate." Biochemistry **28**(15): 6229-36